ORIGINAL ARTICLE

Prevalence of Intestinal Infection due to *Cryptosporidium* Species Among Taiwanese Patients with Human Immunodeficiency Virus Infection

Chien-Ching Hung,[1] John C. Tsaihong,[2] Ya-Tien Lee,[2] Hung-Yin Deng,[3] Wei-Hung Hsiao,[3] Sui-Yuan Chang,[4] Shan-Chwen Chang,[1] Kua-Eyre Su[5]*

Background/Purpose: Cryptosporidiosis causes significant morbidity and mortality in human immunodeficiency virus (HIV)-infected patients who do not receive highly active antiretroviral therapy. Related data on cryptosporidiosis in Taiwanese HIV-infected patients are very limited. This study assessed the prevalence of intestinal infection due to *Cryptosporidium* spp. among Taiwanese patients with HIV infection.
Methods: This retrospective review included 1044 patients with HIV infection treated between June 1994 and June 2004. Intestinal colonization due to *Cryptosporidium* spp. was identified by polymerase chain reaction and restriction fragment length polymorphism of stool specimens collected from 332 of the HIV-infected patients without gastrointestinal symptoms, 90% of whom were receiving highly active antiretroviral therapy.
Results: Five out of 1044 (0.5%) HIV-infected patients had a diagnosis of intestinal cryptosporidiosis by endoscopic biopsy or examinations of stool specimens. Intestinal colonization due to *Cryptosporidium* spp. was found in four of 332 (1.2%) asymptomatic HIV-infected patients between 2001 and 2003; two were due to *C. hominis*, and one each were due to *C. felis* and *C. meleagridis*.
Conclusion: Our findings indicate that the prevalence of intestinal colonization due to *Cryptosporidium* is low among HIV-infected patients in Taiwan. [*J Formos Med Assoc* 2007;106(1):31–35]

Key Words: AIDS, colonization, cryptosporidiosis, *Cryptosporidium parvum*, HIV infection, Taiwan

Cryptosporidia are an important etiology of enteric infections in patients with human immunodeficiency virus (HIV) infection, and cryptosporidiosis, either intestinal or extraintestinal, is associated with a shorter survival.[1,2] The prevalence of cryptosporidiosis in HIV-infected patients with diarrhea has been reported to range from 3% to 16% in developed countries, depending on the population studied, degree of immunosuppression, and use of antiretroviral therapy.[3–5]

With the introduction of highly active antiretroviral therapy (HAART), the incidence of cryptosporidiosis has declined,[6,7] and chronic diarrhea and cryptosporidial infection often resolves with increases in CD4 lymphocyte count.[8–10] However, cryptosporidiosis still occurs in patients

[1]Department of Internal Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, [2]Institute of Tropical Medicine, National Yang-Ming University School of Medicine, [3]Center for Disease Control, Taiwan Department of Health, [4]Department of Clinical Laboratory Sciences and Medical Biotechnology, and [5]Department of Parasitology, National Taiwan University College of Medicine, Taipei, Taiwan.

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***Correspondence to:** Dr Chien-Ching Hung, Department of Internal Medicine, National Taiwan University Hospital, 7 Chung-Shan South Road, Taipei 100, Taiwan.
E-mail: hcc0401@ha.mc.ntu.edu.tw

who continue to have low CD4 count despite HAART.[10]

In Taiwan, cryptosporidia were detected in most of the surface water specimens.[11] However, intestinal cryptosporidiosis has rarely been reported among HIV-infected[12] and HIV-uninfected patients. In this retrospective study, we investigated the prevalence of intestinal cryptosporidiosis by retrospective case review and of intestinal colonization due to *Cryptosporidium* species by means of the polymerase chain reaction (PCR) and PCR–restriction fragment length polymorphism (RFLP) analyses among patients with HIV infection in Taiwan, a country where HIV-infected patients have free universal access to antiretroviral therapy and HIV care.

Materials and Methods

Retrospective case review of enteric cryptosporidiosis

The medical records of 1044 consecutive non-hemophiliac HIV-infected adult patients aged ≥ 15 years seen at the National Taiwan University Hospital between 1994 and 2004 were reviewed. A standardized case record form was used to collect demographic information, clinical and immunologic status of HIV infection, laboratory data, and the presence of intestinal cryptosporidiosis. A standardized protocol was used to investigate the etiologic diagnosis of diarrhea among cases.[13] In brief, at least two stool specimens were obtained for bacterial cultures for patients with diarrhea. Concentrated wet mount preparations of stool specimens were examined by direct microscopy. Fecal smears were stained with modified acid-fast. Upper gastrointestinal endoscopy and colonoscopy and biopsy for histopathologic examination were performed when routine examinations of the stool specimens remained nondiagnostic in patients who had persistent diarrhea. A patient was diagnosed as having intestinal cryptosporidiosis when cryptosporidia were identified in stool or biopsy specimens from patients with diarrhea.

Intestinal cryptosporidial colonization in asymptomatic HIV-infected persons using PCR and PCR-RFLP

Between 2001 and 2003, stool specimens were prospectively collected from 332 HIV-infected patients without diarrhea who were followed at this hospital after diagnosis of HIV infection for investigation of amebic infection.[14] The institutional review board of the hospital approved the study protocol.

Total DNA was isolated from fresh stool specimens by the diatom beads adsorption of nucleic acid in the presence of guanidine thiocyanate and Nonidet P-40 as described elsewhere.[14]

The presence of cryptosporidial nucleic acid in the stool samples was demonstrated by nested PCR developed by Xiao et al[15] with modifications using 18S rRNA gene as the template. Forward primer 5′-TTCTAGAGCTAATACATGCG-3′ and reverse primer 5′-CCCTAATCCTTCGAAACAGGA-3′ were used for the primary amplification. A total volume of 100 μL of reaction mixture containing 0.4 μM primers, 1X PCR buffer, 6 mM $MgCl_2$, 2 μL DNA sample, 0.2 mM dNTP (each) and 2.5 U of *Taq* polymerase (5 U/μL; Invitrogen™ Life Technologies, Brazil) was used. The reaction consisted of 35 cycles of denaturation at 94°C for 45 seconds, annealing at 59°C for 45 seconds, extension at 72°C for 60 seconds, with an initial denaturation at 94°C for 3 minutes and a final extension at 72°C for 7 minutes. In the secondary amplification, forward primer 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and reverse primer 5′-AAGGAGTAAGGAACAACCTCCA-3′ were employed, and the reaction conditions were the same as for the primary reaction, except that 4 mM $MgCl_2$ was used.

For restriction fragment assays, 10 μL each of the secondary PCR products were digested in a 20 μL volume with 5 U of *Ssp* I (New England BioLabs, USA) and *Vsp* I (MBI Fermentas, USA) at 37°C for 1 and 4 hours, respectively. The digested products were fractionated on 3% gel (3:1 Nusieve agarose) and visualized by ethidium bromide staining.

Results

The demographic and clinical characteristics of the two study populations are shown in the Table. Most of the 1044 patients in the retrospective case review were in the late stage of HIV infection at baseline, with a median CD4 count of 81 cells/μL; more than two-thirds of them were diagnosed as having AIDS because of CD4 counts < 200 cells/μL (66.2%) or presence of AIDS-opportunistic illnesses[16] (67.3%) when they first sought HIV care at this hospital. Between 1994 and 2004, only five (0.5%) HIV-infected patients, four males and one female, were diagnosed as having intestinal cryptosporidiosis. Three of them were heterosexuals and two men were homosexuals. All of the five patients had depleted CD4 lymphocyte counts when cryptosporidiosis was diagnosed, with a median CD4 count of 40 cells/μL (mean, 26 cells/μL; range, 1–49 cells/μL). Three cases were diagnosed by acid-fast stained smears of the stool specimens and the other two by endoscopic biopsy of the duodenum. One of the five patients was receiving two nucleoside reverse transcriptase inhibitors when cryptosporidiosis was diagnosed before the introduction of HAART in Taiwan in 1997. He died 4 months later without HAART and anti-cryptosporidial therapy. Of the other four patients who were antiretroviral-naïve, HAART was initiated without anti-cryptosporidial therapy, and all survived as of July 2005. The species of cryptosporidial isolates were not further identified.

The demographic and clinical characteristics of the 332 asymptomatic HIV-infected patients were described previously (Table).[14] In brief, there were 310 males and 22 females, with a median age of 37 years (range, 17–80 years); 62% were men having sex with men; 64.5% had had AIDS-related opportunistic illnesses within 1 month of stool collection. More then 90% of them were receiving HAART and the latest median CD4 lymphocyte count was 265 cells/μL (range, 1–1230 cells/μL); 40% of them had CD4 lymphocyte counts < 200 cells/μL. Of the 332 stool specimens, four (1.2%) were positive for cryptosporidia by PCR (Figure 1). Using nested PCR and RFLP analyses, we were

Table. Baseline characteristics of HIV-infected persons aged ≥ 15 years for retrospective case review and prospective investigation of intestinal infection due to *Cryptosporidium* species*

	Retrospective case review	Prospective survey
Patients (*n*)	1044	332
Age (yr)	34 (15–83)	37 (17–80)
Male gender	92.7	93.4
Risk behavior		
Homosexual/bisexual	61.4	62.1
Heterosexual	31.7	35.2
IDU	2.3	0.9
Others	4.6	1.8
Baseline CD4 count (cells/μL)	81 (0–1202)	265 (1–1230)
< 200	66.2	39.9
200–349	14.6	23.9
≥ 350	19.3	36.2
Baseline PVL (log_{10} copies/mL)	5.17 (2.60–5.88)	2.60 (2.60–5.88)
Naïve to antiretroviral therapy	75.4	9.3
AIDS-OI within 1 mo of enrollment	67.3	64.5
Persons initiating HAART	80.1	90.7

**Data presented as median (range) or %. IDU = injection drug use; PVL = plasma HIV-RNA load by RT-PCR; AIDS-OI = AIDS-defining opportunistic illnesses of CDC[16] plus Penicilliosis marneffei; HAART = highly active antiretroviral therapy.*

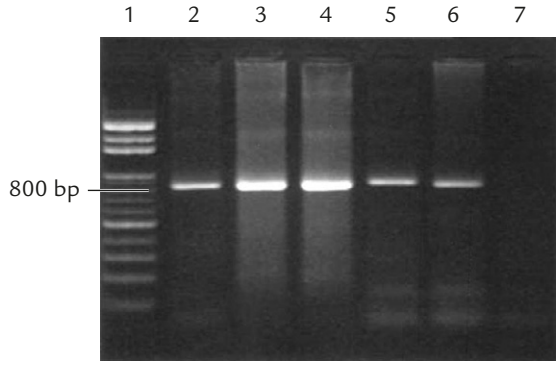

Figure 1. Nested PCR products (826–864 bp) for *Cryptosporidium* spp. SSUrRNA gene sequences in stool specimens from four HIV-infected persons (lanes 3–6). Lane 1 = 100 bp marker; lane 2 = positive control for *C. parvum* bovine genotype; lane 7 = negative control.

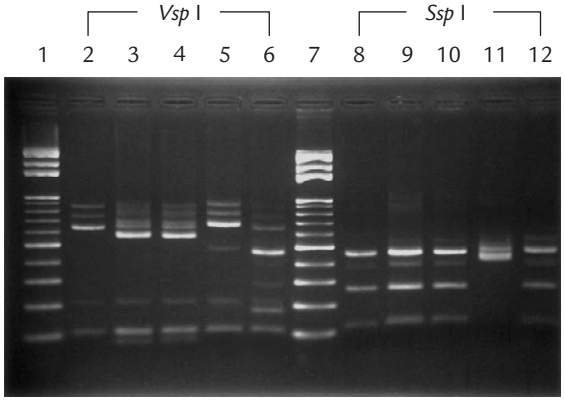

Figure 2. PCR-RFLP of cryptosporidia SSUrRNA gene sequences. Lanes 2–6 and lanes 8–12 show the restriction fragments using *Vsp* I and *Ssp* I, respectively. Lane 2 is the positive control for *C. parvum* bovine genotype with *Vsp* I restriction fragments with sizes of 102, 104 and 628 bp. Lanes 3–6 show the *Vsp* I restriction maps from HIV-infected persons: lanes 3 and 4 = *C. hominis* (70, 102, 104 and 561 bp); lane 5 = *C. felis* (102, 104 and 656 bp); lane 6 = *C. meleagridis* (102, 104, 171 and 456 bp). Lanes 8–12 are the *Ssp* I restriction maps: lane 8 = positive control (108, 254 and 449 bp); lanes 9 and 10 = *C. hominis* (108, 254 and 449 bp); lane 11 = *C. felis* (385 and 448 bp); lane 12 = *C. meleagridis* (108, 254 and 449 bp). Lanes 1 and 7 = 100 bp markers.

able to identify the four isolates as *C. hominis* (2), *C. felis* (1), and *C. meleagridis* (1) (Figure 2). All of the four patients were receiving HAART, and their mean CD4 count had increased to 230 cells/μL when they submitted stool specimens for investigation. None of them had ever been diagnosed as having cryptosporidiosis before.

Discussion

Cryptosporidium spp. appeared to be infrequent etiologies in patients seeking medical care for diarrhea in Taiwan. In a survey investigation using modified acid-fast smears of stool specimens of 1485 patients with diarrhea that was conducted in five major hospitals in Taipei in 1990, only six (0.4%) tested positive for cryptosporidia.[17] In this study, we also found that the prevalence of enteric disease and colonization due to *Cryptosporidium* spp. was low through a 10-year retrospective case review and 3-year prospective survey of stool specimens among HIV-infected patients in Taiwan.

There are several explanations for the low prevalence of enteric disease and colonization due to *Cryptosporidium* spp. among HIV-infected patients in Taiwan. The lower prevalence of infection may be because of lower risk of exposure to contaminated water in Taiwan. Although a high frequency of cryptosporidia can be found in the untreated (77%) and treated (76%) water specimens collected from potable water treatment plants in Taiwan,[11] people in Taiwan are not used to drinking unboiled tap water. Risk of infection due to *Cryptosporidium* spp. can be eliminated by boiling of drinking water.[1]

Under-detection by microscopy might account for the low rate of cryptosporidiosis in HIV-infected patients in our study and for that in the HIV-uninfected patients with diarrhea in Taiwan mentioned previously.[16] However, our step-wise approaches to those immunosuppressed patients with chronic diarrhea through performance of endoscopy and biopsy should be able to provide appropriate diagnostic yields.[13] Though a small sample size, an investigation of stool specimens of 109 asymptomatic school children in mountainous schools in Taiwan using nested PCR did not identify any cryptosporidial infection [Tsaihong, unpublished data].

Risk of infection due to *Cryptosporidium* spp. and development of enteric or extraintestinal cryptosporidiosis in HIV-infected patients depend on the status of immunosuppression.[1,2] HAART restores immunity in the gastrointestinal mucosa

of HIV-infected patients, which may confer those persons protection from cryptosporidial infection and subsequent development of diseases.[7–10] After HIV infection is diagnosed, the majority of our patients initiate HAART with increases of CD4 count. Therefore, the risk for persistent colonization due to cryptosporidia and subsequent development of enteric disease will be significantly reduced.

There are several limitations in our study, and the results should be interpreted with caution. Although the hospital provided both inpatient and outpatient services to HIV-infected patients all around Taiwan, we did not specifically investigate whether the risk for cryptosporidiosis was related to their place of residence because the degree to what extent the surface water was contaminated might be different and their residence might change. This study of identification of cases of cryptosporidiosis in HIV-infected patients was retrospective in study design at a referral center for HIV care; cases of cryptosporidiosis in patients in the earlier stages of HIV infection may not be identified. In the detection of intestinal cryptosporidial colonization, a cross-sectional survey may not be able to detect patients who intermittently shed cryptosporidia and, therefore, the prevalence of cryptosporidial infection may be underestimated. Longitudinal follow-up of HIV-infected patients using PCR and RFLP that is more sensitive than microscopy may provide a more complete insight into the epidemiology of enteric cryptosporidial infection in HIV-infected patients in Taiwan.

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